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Review

Imaging mRNA movement from transcription sites to translation sites

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Abstract

RNA localization is one mechanism to temporally and spatially restrict protein synthesis to specific subcellular compartments in response to extracellular stimuli. To understand the mechanisms of mRNA localization, a number of methods have been developed to follow the path of these molecules in living cells including direct labeling of target mRNAs, the MS2-GFP system, and molecular beacons. We review advances in these methods with the goal of identifying the particular strengths and weaknesses of the various approaches in their ability to follow the movements of mRNAs from transcription sites to translation sites.
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1. Introduction

mRNA localization and localized translation are mechanisms to control gene expression both temporally and spatially within the cell. It is now clear that non-coding sequences contained within the untranslated regions (UTRs) of many mRNAs serve as information for the specific placement of that transcript within the cytoplasm and for the timing of its translation. The life of a localized protein begins in the nucleus at the site of transcription. The nascent mRNA is co-transcriptionally packaged with *trans*-acting proteins into messenger ribonucleoprotein particle (mRNP) and is subsequently exported from the nucleus through nuclear pores. In the cytoplasm the complement of proteins associated with the mRNP is remodeled and the mRNP is then delivered to its target cytoplasmic destination by several mechanisms [1,2]. At the target site the mRNP is anchored and upon receiving the appropriate signal, the complex is once again remodeled to relieve translational repression and the mRNA is locally translated into protein [3]. Recent advances in the development of fluorescence-based methods to follow single mRNAs and the complexes they form in living cells has shed light on a number of the steps in the life of a peripherally localized mRNA. Here we provide a review of the methods used to follow mRNAs from their sites of transcription to their sites of translation and discuss the mechanisms of mRNA localization that have been gleaned from these approaches.

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2. Imaging specific mRNAs in living cells

A number of technical issues must be considered when designing a method to image specific mRNAs in living cells. A critical technical hurdle to overcome is the need to maximize the signal to noise ratio of the optical system while minimizing phototoxicity to samples. An additional requirement is that images must be acquired at a rate at least twice as fast as an object moves to an adjacent pixel. These challenges are effectively overcome by utilizing microscopes equipped with wide-field optics coupled to high-speed, sensitive cooled EM-CCD cameras. An additional technical constraint to consider is how to label a specific mRNA in the context of numerous RNAs without increasing the noise of the optical system. Once these issues are addressed, there remains the need to introduce a reporter into the cell with minimal perturbation to cellular structure and function. An additional consideration is whether the reporter mRNA will be properly recognized by the transport machinery, since it has been noted that some mRNAs must be processed in the nucleus before subsequent cytoplasmic events can occur properly [4]. A number of methods exist that effectively balance these issues allowing investigators a glimpse of the dynamics of mRNA targeting and transport.

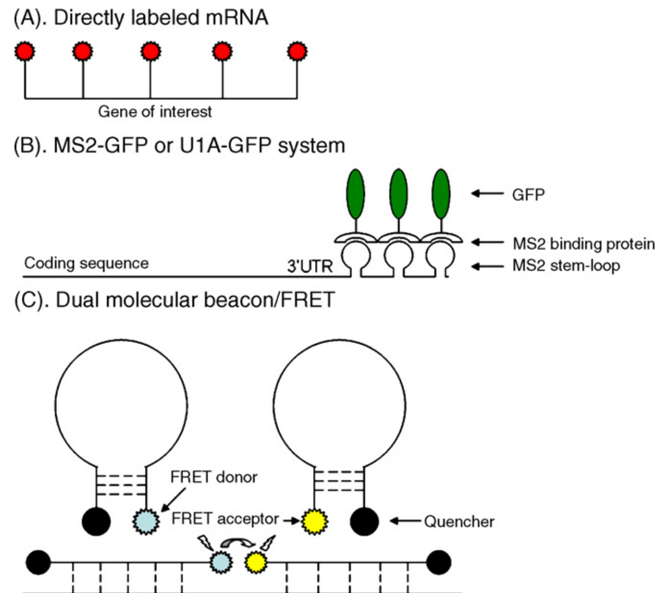

Fig. 1. Schematic representation of some the methods for visualizing mRNA movements in living cells. (A) Direct labeling of an mRNA with multiple fluorophores. (B) Stem-loop/stem-loop-binding-GFP fusion protein system. (C) Dual molecular beacon/FRET system. The dashed lines represent standard Watson/Crick base pairs.

3. MS2-GFP labeling of mRNA

To investigate the dynamics of mRNA movement, it was necessary to develop methods to track specific mRNAs in real time in living cells. One method was developed utilizing the high affinity interaction between sequence-specific RNA stem-loops and the bacteriophage capsid protein MS2 [5]. Incorporation of multiple repeats of the MS2 stem-loops into an RNA sequence of interest creates an interaction platform capable of binding to multiple MS2 proteins each fused to GFP (Fig. 1). Twenty-four repeats were found to be sufficient to detect single mRNA molecules [6]. The simultaneous expression of a stem-loop-containing mRNA and the MS2-GFP in living cells provides a powerful method for detecting specific mRNP complexes [5]. An elegant solution to the signal to noise problem for tracking specific mRNAs within the cytoplasm is provided by having an NLS contained within the MS2-GFP protein that sequesters it within the nucleus when not bound to an MS2-containing RNA target. The high affinity (∼1 nM) interaction between the stem-loop sites and the MS2 protein ensures that most reporter mRNAs are bound by a number of MS2-GFP fusion proteins and that the majority of GFP signal emanates from *bona fide* target transcripts. Since these reporter molecules are encoded within plasmid vectors, they can be transfected into the cell before the experiment is performed, minimizing perturbations to cellular structure and function associated with microinjection. Driving the expression of the reporter with an endogenous promoter can result in more physiological levels of the mRNA of interest. Importantly, the mRNAs generated from this reporter are transcribed in the nucleus and are properly packaged, exported, targeted, and translated, making MS2-GFP a good system to track mRNAs from their sites of synthesis to translation.

Imaging within the nucleus demonstrated the utility of MS2-GFP in the identification [7,8] and characterization of transcription sites [9]. Tracking the movement of mRNA within the nucleus indicated that diffusion is the primary mechanism by which these molecules translocate from transcription sites to the nuclear periphery [8]. Within the cytoplasm the types of movement exhibited by mRNAs are more complicated. Using the MS2-GFP system and imaging the cytoplasm it has been shown that mRNAs exhibited directed, corralled, diffusive and static movements [6]. The velocities, direction, *cis*-elements and *trans*-acting factors required for these movements have been characterized utilizing the MS2-GFP system [5,6]. In *Drosophila* oocytes two novel mechanisms for mRNA targeting within the cytoplasm, diffusion and entrapment and continual active transport, have been revealed using MS2-GFP. Hence MS2-GFP has been shown to have single molecule sensitivity and has been used to track mRNAs within both the cytoplasm and the nucleus.

MS2-GFP has been successfully applied to tracking specific cytoplasmic and nuclear mRNAs in yeast, *Dictyostelium*, plants, flies, and mammalian cells [5,6,10–13]. For example, this system has been used to show that *ASH1* mRNA is localized to the bud tip in *S. cerevisiae* in a zipcode-dependent manner. Zipcodes are *cis*-acting sequences often contained within 3′UTRs of mRNAs that specify the spatial information for mRNA targeting to a specific cellular location through their affinity to mRNA-binding proteins that interact with cytoskeletal components and organelles [3]. It was demonstrated that proper *ASH1* mRNA targeting required the expression of the SHE proteins. She1p is a yeast homologue of the mammalian class 5 myosin motor proteins and was required for bud tip localization along with the She2/She3 proteins, a complex which is the only example of a

direct link between a localized mRNA and a molecular motor [14]. Time-lapse imaging revealed that labeled mRNAs move to the bud tip at a rate of 0.2–0.44 μm/s in a directed fashion [5,10]. In living mammalian cells, MS2-GFP was used to show that cytoplasmic mRNAs exhibit directed, corralled, diffusive, and static movements. The *β-actin* zipcode increased the number and persistence of these directed movements relative to a non-zipcode containing control reporter. Single particle tracking and time-lapse imaging revealed directed mRNA movements along microtubules at an average rate of 1–1.5 μm/s [6]. Of particular interest was the observation that translating mRNAs are associated with cytoskeletal filaments and appear to be a subset of the static mRNAs [15]. Consistent with these observations, mRNA movements in rice endosperm cells also exhibited static, directed, and corralled movements. The average velocity for the directed movements was between 0.3 and 0.4 μm/s. These movements required intact actin filaments as they were abrogated by latrunculin B or cytochalasin D treatment [12]. By contrast, when MS2-GFP was used to track *nanos* mRNA during *Drosophila* oogenesis it was demonstrated that a diffusion and entrapment mechanism was utilized to accumulate this mRNA at the posterior of the developing oocyte. This mechanism required intact actin filaments as depolymerization of actin resulted in a loss of both the mRNA and the pole plasm from the posterior pole suggesting each is anchored to the cytoskeleton [11]. Additional work in *Drosophila* tracking *bicoid* mRNA movements revealed a novel continual active transport model for the anterior localization of this transcript. These movements were both microtubule- and dynein-dependent as colcemid treatment or dynein heavy chain mutants abrogated the localization to the anterior of the egg chamber. Single particle tracking analysis of stage 13 egg chambers reveals anterior directed movements at rates ranging between 0.03 and 0.12 μm/s [13].

Rapid analysis of specific mRNA trafficking dynamics in living neurons became possible utilizing MS2-GFP labeling. The first mRNA analyzed in neurons with this system was *CaMKIIα*, a highly abundant dendritic mRNA encoding a protein involved in the establishment and maintenance of synaptic plasticity in the hippocampus. Cultured neurons transfected with this mRNA showed particles that moved with both oscillatory and persistent trajectories, both in the anterograde and retrograde directions, and neuronal activity modified these dynamics. Neuronal depolarization increased the numbers of granules in dendrites and also increased the fraction of *CaMKIIα* mRNA particles moving in the anterograde direction. In addition, this activity caused a repositioning of the population of mRNAs already localized to dendrites with respect to synapses, resulting in an enhancement of the mRNA at synaptic sites. Quantification of mRNA granule dynamics showed that average transport rates were in the range of 0.05 μm/s for persistent trajectories and maximal rates at up to 0.2 μm/s [16]. Interestingly, a recent report using much faster sampling rates than those used previously showed that *CaMKIIα* mRNA moved at velocities approximately ten-fold faster, on the order of 0.5 μm/s on average and up to 2.0 μm/s maximally [17]. These data suggest that mRNA dynamics are responsive to synaptic activity and that current technology for tracking mRNA movements in living cells is limited by camera sampling rates in addition to signal to noise levels.

The MS2-GFP system has also been successfully applied to studies of mRNA movements occurring within the nucleus. Nascent mRNAs were detected through the spatial amplification of signals provided by the multiple (33) MS2-GFP-bound mRNAs undergoing the process of transcription [7–9]. Tagging discoidin I, a developmentally regulated gene, in *Dictyostelium* revealed that transcription of this gene occurs in pulses with a mean duration of ∼5 min. Surprisingly, monitoring the transcriptional status of large numbers of cells in real-time revealed a form of "transcriptional memory" where the probability of a transcriptional pulse for a particular gene is increased in those cells that have exhibited previous transcriptional activity of this gene. In addition, these studies revealed that transcriptional pulses occur within clusters of cells suggesting local cues for transcriptional activation or repression [9]. The MS2-GFP system has also been used to follow a reporter mRNA from transcription sites through the nucleoplasm. The movement of these mRNAs was shown to occur through simple diffusion by utilizing single particle tracking, fluorescence recovery after photobleaching (FRAP), and local photoactivation [8]. The mRNPs moved an average of 5 μm at velocities ranging from 0.03 to 0.08 μm/s. These movements were not affected by the metabolic inhibitors 2-deoxyglucose or sodium azide, which rules out active transport or local anchoring at chromatin that requires ATP [8], and were consistent with diffusional rates. These findings establish a model by which mRNAs move from transcription sites to nuclear pores primarily by diffusion and do not bind to structural nuclear components en route to nuclear pores.

4. U1Ap-GFP system

The concept of incorporating a specific RNA aptamer into a gene of interest that can be recognized by a GFP-aptamer-binding protein fusion led to the creation of the U1Ap-GFP system as an alternative to MS2. By adding multiple repeats of the U1A splicing protein recognition sequence into a gene of interest and co-transfecting an U1Ap-GFP fusion protein, investigators have been able to track the movements of mRNA in yeast cells [18–20]. The strengths and weaknesses of this approach are identical to those of the MS2-GFP system with the caveat that it can only be used in yeast cells since mammalian cells have endogenous U1A. Following mRNA movements from transcription to translation sites is also possible using the U1Ap-GFP system. This imaging system allows for the tracking of specific mRNAs within a genetically tractable cell type. For example, nuclear export of mRNAs is blocked in yeast strains with mutations to nuclear export factors [19]. Within the cytoplasm, U1Ap-GFP has been utilized to demonstrate directed movements and colocalization between an mRNA and the *trans*-acting factors required for its localization [18]. Most recently, U1Ap-GFP-labeled mRNAs have been shown to move into and out of P-bodies, putative sites of mRNA decapping and degradation [20]. These findings demonstrate the utility of live imaging to better understand the dynamics of enchange between distinct mRNA compartments, and suggest that some mRNAs marked

for degradation may be rescued and recycled from P-bodies. This system is ideally suited to determine the signaling components that may play a role in regulated translation or degradation of localized mRNAs.

Using the U1Ap-GFP system on *ASH1* mRNA confirmed that this mRNA was localized to the bud tip in *S. cerevisiae*. Imaging of U1Ap-GFP-labeled *ASH1* mRNA with SHE proteins, polypeptides required for *ASH1* mRNA localization, revealed that She2p, She3p, and Myo4p (She1p) co-localized with *ASH1* mRNA [18]. U1Ap-GFP has also been used to track *PGK1* and *MFA2p* mRNAs into and out of P-bodies in the absence or presence of glucose, respectively. The movement of these mRNAs from P-bodies required translation initiation factors as these translocations failed to occur in mutant strains lacking eukaryotic initiation factor 3 [20]. This result suggests that there is a dynamic exchange of mRNA between P-bodies and polysomes with the distribution correlating with the translational status of the mRNA. Thus, in addition to their role in decapping and degradation, P-bodies may also function as storage sites for mRNA during times of cellular stress. It is tempting to speculate that mRNA movements through P-bodies may be an initial step en route to localized translation, assembling NMD factors (such as Upf1) that are markers for the pioneer round of translation and enabling function as a sensor of mRNA translatability [21]. Interestingly, Upf1 has been shown to be involved Staufen1-mediated mRNA decay that is a distinct pathway from NMD-mediated decay, and therefore future studies looking at the movements of Staufen1 and Upf1 with mRNA targets in living cells may be very revealing.

U1Ap-GFP has also been applied to study nuclear export in living yeast cells. Reporter mRNAs comprising the *PGK1* ORF, the *ASH1* or the *PGK1* 3′UTR failed to exit the nucleus in conditional mutant strains, e.g. *mex67-5* or *xpo1-1*, where nuclear export is blocked. Mutations in factors involved in the regulation of the Ran GTPase also caused nuclear accumulation of these reporter mRNAs. In addition, it was shown that mutations to components of the splicing machinery, such as prp 22-1 or prp 16-2, also led to the nuclear accumulation of intron-containing mRNAs while not affecting intron-less mRNAs. Finally, it was demonstrated that mutations in 3′ processing factors, including PAP1, also prevented nuclear export of mRNAs [19].

5. Directly labeled mRNAs

Imaging directly labeled mRNAs addresses two of the major issues with live cell imaging. First, direct labeling of the mRNA ensures that the observed fluorescence signal only comes from the mRNA of interest because the fluorophores are attached to the reporter prior to its introduction into the cell providing exceptional specificity (Fig. 1). An important consideration with this method is that the mRNP that is formed when the mRNA is injected into the cytoplasm has not been exposed to the nucleus, and may lack nuclear factors important for localization. This assay has been used to assess the nuclear factors needed by mRNAs for their proper targeting by injecting a fluorescent mRNA into the nucleus, removing the nucleoplasm, and subsequently injecting it into the cytoplasm of a different cell to observe its movement [22]. Imaging directly labeled mRNAs provides good temporal resolution because the observed fluorescence signal is present immediately and does not require hybridization of a probe to the reporter or folding of GFP. A potential caveat of this technique is that the labeled mRNAs are often injected into cells which can perturb the physiological state of the cells. Moreover, one must consider whether the localization pattern observed with directly labeled mRNAs accurately reflects that of endogenous mRNAs since it is possible to titrate out factors of the endogenous localization machinery. Finally, the RNAs injected are usually orders of magnitude higher in abundance than the endogenous RNA and this may create artifacts.

Imaging directly labeled mRNA confirms that movement within the nucleus occurs primarily via diffusion [23]. Within the cytoplasm the story is more complicated. Some directly labeled mRNAs translocate as granules exhibiting directed movement [24] while others are localized via cytoplasmic streaming and anchoring [25]. An additional area where directly labeled mRNAs are powerful research tools is for studying the potential *trans*-acting factors required for proper transcript targeting. Directly labeled mRNAs were used to show that *bicoid* (*bcd*) mRNA requires factors present in *Drosophila* nurse cells for proper anterior targeting in egg chambers [22]. Thus, directly labeled mRNAs are good tools for studying the movements of specific transcripts and the required *trans*-acting factors for said movements as long as the factors are in excess.

Fluorescent *myelin basic protein* mRNA microinjected into oligodendrocytes formed granules throughout the cytoplasm that exhibited persistent directional movements with a velocity of 0.2 μm/s from the cell body through processes and finally accumulated in the myelin compartment. Additional oscillatory movements were observed at cytoskeletal branch points with a mean displacement of ∼0.1 μm/s [24]. During stage 10 and 11 of *Drosophila* oogenesis, microinjected *oskar* mRNAs formed granules that were localized by cytoplasmic streaming and subsequent association with a posterior anchor. In contrast, microinjected *bicoid* mRNA was localized throughout the oocyte cortex. The localization of *oskar* by cytoplasmic streaming was shown to be distance-dependent as short range movements were achieved in the absence of streaming [25]. Alexa-labeled *runt* (*run*), *fushi tarazu* (*ftz*), and *wingless* (*wg*) are all apically targeted when injected into the basal cytoplasm or yolk. Each transcript accumulates into granules within 30 s that move directly to the apical cytoplasm of *Drosophila* blastoderm embryos at a rate of 0.5 μm/s. Simultaneous injection of two apically localized transcripts revealed that the granules may contain multiple mRNAs. Interestingly, when apical transcripts were simultaneously injected with basally targeted or diffuse transcripts, there was no observed colocalization within the granules suggesting that each granule contains transcripts targeted to different destinations, and that *cis*-acting information on the mRNA may encode granule assembly as well as localization information. Colcemid treatment prior to injection of the apically-targeted transcripts prevented their localiza-

tion, providing evidence that apical targeting in *Drosophila* blastoderm embryos is microtubule-dependent. Furthermore, pre-incubation with antibodies against the dynein heavy chain prevented apically-targeted transcripts from properly reaching their targets, highlighting a dynein-dependent step in apical localization and a novel function for dynein in mRNA anchoring [26,27]. Interesting data concerning the transport of *bcd* mRNA from nurse cells to the oocyte has been generated through imaging injected *bcd* in living egg chambers. When FITC-labeled *bcd* transcripts were injected into nurse cell cytoplasm, the mRNA localized to the anterior of the oocyte suggesting that all of the factors required for this localization pathway are contained within the nurse cell cytoplasm. This localization was disrupted when a *bcd* transcript with a deletion of the bicoid localization element 1 in the 3′UTR was injected into nurse cells. This pathway was dependent on microtubules as localization of injected *bcd* was prevented in the presence of colcemid. Time-lapse imaging revealed that the injected *bcd* mRNA moved at a rate of ~1.5 μm/s. Of particular interest was the requirement for factors in the nurse cell for the proper anterior localization of *bcd*. When labeled *bcd* was injected directly into the oocyte, the mRNA was localized to the nearest cortical surface. By contrast, when the labeled *bcd* was first injected into nurse cells and then removed and injected into the oocyte, the mRNA exhibited the proper anterior localization [22].

Another use of fluorescent reporter mRNAs in neurons comes from studies on the role of the cytoplasmic polyadenylation element (CPE) in dendritic mRNA localization [28]. Originally identified as a 3′UTR sequence that controls cytoplasmic regulation of polyadenylation of mRNAs and subsequent translation, CPE-containing mRNAs are bound by a family of RNA-binding proteins termed CPEBs that mediate specific interactions with proteins of the translational machinery in eukaryotic cells. Co-injection of a CPE-containing fluorescent-labeled mRNA reporter and a plasmid bearing the CPEB-GFP into B104 neuroblastoma cell lines demonstrated colocalization between the mRNA and its binding protein. Both appear as dendritic granules that moved in a microtubule-dependent manner. This localization depended both on the CPE and on the cognate RNA-binding protein CPEB, as CPE-lacking mRNAs showed reduced colocalization with co-injected GFP-CPEB and deletion of the CPEB1 gene caused a reduction in fluorescent CPE-reporter mRNA localization to dendrites [29].

The dynamics of mRNA movement within the nucleus have also been studied utilizing fluorescently labeled, *in vitro* synthesized mRNAs confirming that these movements occur primarily through diffusion-based mechanisms. Cy3-labeled *β-globin* and *EGFP* mRNAs were microinjected into *Xenopus* A6 cells forming fluorescent granules throughout the nucleus. Single particle tracking of these transcripts revealed that approximately 50% of the particles were moving at any given moment with diffusion coefficients of 0.21 and 0.18 $\mu m^2/s$ for *β-globin* and *EGFP*, respectively. In addition, these movements were unaffected by energy depletion by sodium azide and 2-deoxyglucose confirming that they were energy independent [23].

6. Molecular beacons

An elegant solution to the issues inherent in imaging and tracking individual mRNAs is provided by the use of molecular beacons [30]. Molecular beacons are reporter molecules containing a fluorophore on one end and a quencher on the other end with a short stem-loop structure (Fig. 1). This prevents these molecules from generating fluorescence until they hybridize with their target mRNA. A significant improvement in signal to noise and specificity can be achieved by the simultaneous expression of two molecular beacons containing fluorophores that are good FRET pairs (Fig. 1). Each molecular beacon is designed such that their hybridization sequences are complementary to nearly adjacent (~10 nt) sequences within the target mRNA. When the molecular beacon hybridizing to the 5′ end of the target has its fluorophore on its 3′ end and the other beacon has its fluorophore on its 5′ end, energy transfer between the reporters can only occur when both are hybridized to the target simultaneously. This setup addresses some of the initial shortcomings associated with the use of molecular beacons, such as identifying false positive signals caused by spontaneous unfolding or partial degradation of the reporter. At present, delivery of molecular beacons by microinjection, transfection, or coupling to cell-penetrating peptides results in rapid accumulation within the nucleus complicating the detection of cytoplasmic mRNAs. This has been addressed by adding bulky proteins, such as streptavidin, to the reporters or by fusing the molecular beacons to molecules resident in the cytoplasm, such as tRNA transcripts [31]. Currently, this approach suffers from the need for two hybridization events to identify the mRNA of interest lowering the efficiency of the technique. Moreover, information on the tertiary structure and the sequences bound by RNA-binding proteins on the target mRNA is required to select stretches where hybridization between the molecular beacon and the mRNA will be favored and does not interfere with essential signals in the target RNA. Finally, it is critical to assess the effect of the hybridization of molecular beacons on the physiology of the cell to ensure that it does not result in double strand-mediated degradation of the target or translational silencing.

Imaging cells containing molecular beacons has been used to characterized a number of steps in the travels of mRNAs from transcription to translation sites. Transcription sites have been identified via this technique. Within the cytoplasm molecular beacons have been useful in confirming the posterior localization of *oskar* in stage 9 and 10 *Drosophila* egg chambers. In fact, a beacon microinjected into the nurse cell cytoplasm was followed to the posterior of an egg chamber [32]. In mammalian cells a beacon complementary to *β-actin* mRNA was tracked from the perinuclear cytoplasm toward a lamellipodium following serum induction [30]. These data established molecular beacons as an additional technique capable of following mRNA movements in living cells.

Dual molecular beacons and FRET were utilized in living cells to demonstrate that heterologous *c-fos* mRNA is found throughout the cytoplasm of Cos7 cells [33]. In addition, molecular beacons were used to estimate the relative expression levels of *c-fos* in cells demonstrating that this optical technique was

in good agreement with data obtained by dot blotting experiments [33]. The dual molecular beacon technique was used to show *K-ras* mRNAs were not diffusely distributed but were localized throughout the cytoplasm in a cable-like distribution reminiscent of microtubules in normal human dermal fibroblast cells, suggesting that this transcript may be associated with the cytoskeleton [34]. By contrast, when the authors used dual molecular beacons and FRET to investigate the distribution of *survivin* mRNA, the pattern was different with the majority of the signal exhibiting an asymmetric distribution on one side of the nucleus in MIAPaCa-2 pancreatic carcinoma cells [34]. Further studies utilizing dual molecular beacons revealed that *K-ras* and *GAPDH* mRNA co-localize with a marker for mitochondria in HDF cells [31].

In an alternate approach, dual molecular beacons where one beacon contains sequences complementary to the target mRNA and the other beacon contains sequences that will not bind to the target can be used to show that the distribution of the complementary beacon is specific for the target sequence. In this case the ratio of the specific to non-specific beacon is calculated and areas with a high value ratio are representative of localisation sites of the target mRNA. This corrects for the signal that is generated from non-specific localization of the molecular beacon. This approach was utilized to confirm that *oskar* mRNA is targeted to the posterior pole of stage 9 and 10 oocytes in *Drosophila* in a microtubule-dependant manner. Utilizing the alternative dual molecular beacon approach allowed the authors to demonstrate that when the reporter is microinjected into nurse cells *oskar* traffics to the posterior pole of the oocyte within 90 min indicating that molecular beacons can be used to follow the travels of a specific mRNA in living cells [32]. The distribution of *β-actin* mRNA in fibroblasts was determined utilizing a molecular beacon specific for this mRNA and a second molecular beacon with a non-specific sequence. The ratio between these molecular beacons demonstrated that *β-actin* was localized to active lamellipodia. In addition, the authors showed that there was a flow of *β-actin* from the nucleus towards a lamellipodium after only 2 min of serum stimulation. A time-lapse movie showed the movement of *β-actin* mRNA from an old lamellipodium to a new lamellipodium, effectively demonstrating the high temporal and spatial resolution that can be achieved through the use of molecular beacons. Of particular interest, microinjection of preformed complexes between a molecular beacon and the coding region of GFP failed to prevent the translation of the GFP, suggesting that the hybridization of molecular beacons to their target mRNAs does not interfere with translation [30].

Molecular beacons are also useful in identifying transcription sites within the nuclei of living cells. When a molecular beacon complementary to the coding sequence of *human cytomegalovirus immediate early antigen* mRNA was injected into transformed rat fibroblast R9G cells, single bright foci were observed within the nucleus [35].

7. Conclusions and prospects

In the past decade, there has been considerable progress in the ability to track individual mRNAs in living cells. Each method described in this review can be used to track mRNA movements in living cells (Table 1). Direct labeling of mRNAs (Fig. 1A) is easy to use and provides a high signal to noise ratio and specificity with the caveat that this method can only assess transport steps in the cytoplasm since the reporter is not transcribed and thus does not undergo the normal nuclear processing steps of endogenous mRNAs. In contrast, the MS2-GFP and U1A-GFP systems (Fig. 1B) are genetically encoded, start out as transcribed reporters, and can be followed all the way from transcription to translation sites. These systems have high signal to noise ratios and specificity but suffer from the large size of the mRNPs that are formed. A single mRNA has on average 33 GFP molecules bound to 24 (bipartite) MS2 repeats [6] contained in

Table 1
Comparison of different methods to image mRNA movements in living cells

	Directly labeled mRNA	MS2-GFP or U1A-GFP	Molecular beacons with FRET
Delivery method	Microinjection or transfection	Transfection	Microinjection, transfection or peptide-linked
Signal to noise	Excellent (based on the number of fluorophores/reporter)	Good (with 24 MS2 repeats and an NLS in the MS2-GFP fusion possible to detect single cytoplasmic mRNAs); however free GFP increases background	Good (false positives low due to use of FRET)
Specificity	Excellent (essentially all of the signal comes from the mRNA of interest)	Good (interaction between MS2-GFP and the MS2 stem-loops has high affinity)	Excellent (FRET between beacons requires two hybridization events)
Strength	Easy to generate and interpret data	Capable of following mRNA in both the nucleus and cytoplasm; excellent system to track mRNAs from transcription to translation; can achieve high signal to noise	Highly specific
Drawbacks	mRNA delivered without bound *trans*-acting proteins; reporter not transcribed, i.e. no nuclear processing; high concentrations of reporter may overwhelm endogenous trafficking machinery; microinjection can cause severe cell damage	Size of mRNP complex (with the added stem-loops and GFPs the reporter is very large)	Complicated data analysis is required as well as two separate hybridization events

the reporter, making MS2-GFP reporters large relative to other reporters capable of tracking mRNA in living cells. FRET imaging of dual molecular beacons (Fig. 1C) has a high signal to noise ratio and specificity. Tracking cytoplasmic mRNA movements has been achieved using dual molecular beacons. Unfortunately, this system necessitates multiple hybridization events, which requires some time and makes detection of transcription sites difficult because transcription occurs faster than the signal generation. Furthermore, the "breathing" of the beacon creates a background so that the signal to noise ratio rarely exceeds 5:1. For example, molecular beacons have been employed to detect highly abundant *β-actin* mRNA in living cells [30]. However, as noted by the authors of this study, they achieved a signal of only 2.5-fold higher than the background fluorescence.

At present, there is still a need to increase the signal to noise ratio to follow individual mRNAs with faster image capture rates for more accurate velocity measurements. Further development of methods to follow multiple individual mRNAs simultaneously would help assess the relationship between the mRNAs that code for components of multi-protein complexes, allowing investigators to connect mRNA trafficking pathways with cell physiology. Moreover, dual labeling using RNA-binding proteins in conjunction with the RNA tracking methods would enable the visualization of the assembly of functional RNA-protein complexes. Continued work in this field will help investigators better understand how the dynamics of localized gene expression can directly affect diverse aspects of cell physiology.

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